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Colors and pterin pigmentation of pierid butterfly wings

B. Wijnen, H.L. Leertouwer, D.G. Stavenga*

Department of Neurobiophysics, University of Groningen, Nijenborgh 4, NL-9747 AG Groningen, The Netherlands

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Abstract

The reflectance of pierid butterfly wings is principally determined by the incoherent scattering of incident light and the absorption by pterin pigments in the scale structures. Coherent scattering causing iridescence is frequently encountered in the dorsal wings or wing tips of male pierids. We investigated the effect of the pterins on wing reflectance by local extraction of the pigments with aqueous ammonia and simultaneous spectrophotometric measurements. The ultraviolet-absorbing leucopterin was extracted prominently from the white *Pieris* species, and the violet-absorbing xanthopterin and blue-absorbing erythropterin were mainly derived from the yellow- and orange-colored Coliadinae, but they were also extracted from the dorsal wing tips of many male Pierinae. Absorption spectra deduced from wing reflectance spectra distinctly diverge from the absorption spectra of the extracted pigments, which indicate that when embedded in wing scales the pterins differ from those in solution. The evolution of pierid wing coloration is discussed.

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Keywords: Coliadinae; Pierinae; *Colotis*; Wing reflectance; Animal coloration

1. Introduction

Butterflies are some of the most eye catching animals, due to their vividly colorful wings, where stacks of colored scales together form patterns, characteristic for each species (Nijhout, 1991; Kinoshita and Yoshioka, 2005). These colors are either due to pigments that absorb light in a restricted part of the visible wavelength range or due to structures that reflect light in a specific wavelength band. The optics of butterfly coloration is far from understood in detail, however, as almost every species and certainly those in different families apply a variety of optical coloring methods; often pigmentary and structural coloration techniques are combined in a non-trivial way (Kinoshita and Yoshioka, 2005; Stavenga et al., 2006). Especially in those cases where butterfly scales feature so-called photonic crystals, quantitative descriptions are still in their infancy (Vukusic and Sambles, 2003; Kinoshita and Yoshioka, 2005; Michielsen and Stavenga, 2007). For the study of butterfly coloration, a relatively simple and therefore attractive case is presented by members of the Pieridae

(Kemp et al., 2005), a family of butterflies that have brightly colored wings, characterized by large, bold patterning. The two main subfamilies of the Pieridae are the Coliadinae or sulfurs, which mostly are yellow or orange, and the Pierinae or whites, whose wings are predominantly white.

A butterfly scale typically consists of a highly structured upper lamina and a rather flat, unstructured lower lamina. The numerous longitudinal ridges of the upper lamina rest on the lower lamina by pillars or trabeculae and are connected by crossribs (Ghiradella, 1998). The scale material, cuticle, has a refractive index very different from that of air, so that incident light is scattered by the scale structures; the back-scattering determines the reflection, the forward scattering the transmission.

Generally, each side of a wing has two overlapping layers of scales, the cover and ground scales. Incident light is partly reflected and transmitted by each scale layer, so that the reflectance spectrum of a wing is the cumulative result of reflection and transmission in the scale stacks including the wing substrate (Vukusic et al., 1999; Yoshioka and Kinoshita, 2006; Stavenga et al., 2006). In some cases, notably in the cover scales on the dorsal wings of male Coliadinae and on the dorsal wing tips of males of *Colotis*

*Corresponding author. Tel.: +31 50 363 4785; fax: +31 50 363 4740.

E-mail address: D.g.stavenga@rug.nl (D.G. Stavenga).

species, the longitudinal ridges are elaborated with lamellae in a spatially periodical manner. Light scattering by the lamellae is then coherent, resulting in iridescence, which is usually invisible for the human eye (but not for butterfly eyes), as predominantly ultraviolet light is reflected (Ghiradella et al., 1972). The wings of female pierids are generally non-iridescent, and consequently many pierid species have a marked sexual dichromatism (Silberglied and Taylor, 1973; Kemp et al., 2005; Rutowski et al., 2007). The physical basis of the sexual dichromatism of many species in the Coliadinae and *Colotis* group, hence, is sex-dependent coherent light scattering.

Light scattering is generally incoherent, however, resulting in a rather diffuse reflection. When the scales contain pigment, their reflectance is suppressed in the wavelength range of pigment absorption. For instance, black (or brown) scales contain melanin, which absorbs throughout the visible wavelength range, including the ultraviolet. The melanin is dispersed in the ridges and crossribs (Stavenga et al., 2004). The pigments responsible for the non-black wing areas of pierids have been analyzed by extracting them with chemical methods (Hopkins, 1895; Makino et al., 1952; Watt, 1964; Descimon, 1971). The pigments all appeared to belong to one class, appropriately called the pterins (Descimon, 1975; Kayser, 1985). Leucopterin and isoxanthopterin absorb exclusively in the ultraviolet, and consequently inhomogeneous, scattering substances containing these pterins have a white color; xanthopterin and dihydroxanthopterin absorb in the violet as well, and thus produce a yellow color; and with the blue-absorbing erythropterin an orange or red color remains (Pfleiderer, 1963; Descimon, 1971; Watt, 1972).

The pterin pigments are concentrated in pigment granules that adorn the crossribs, a special property of pierid wing scales (Yagi, 1954; Ghiradella et al., 1972; Ghiradella, 1998; Morehouse et al., 2007). The pigment granules, also called beads, have a dual role, because they absorb light in the wavelength range of the pigment absorption spectrum, but in addition they strongly scatter light in the complementary wavelength range (Stavenga et al., 2004; Morehouse et al., 2007). For instance, the male small white, *Pieris rapae crucivora*, has wing scales with a high density of beads, which contain ultraviolet-absorbing pigment. This results in a low reflectance of the wings in the UV, and a high reflectance in the visible wavelength range. The wing scales of the female small white, *P. r. crucivora*, have virtually no beads, however, and consequently the wing reflectance in the UV is much higher than that of the male, but the reflectance at the visible wavelengths is much lower (Obara and Majerus, 2000; Giraldo and Stavenga, 2007). The physical basis of the sexual dichromatism of *P. r. crucivora* (and other related pierids; e.g., Morehouse et al., 2007) therefore is sex-dependent incoherent light scattering.

The structural and pigmentary basis for the colors of pierids hence is in principle clear, but a quantitative understanding of pierid wing reflectance has not yet been reached (for a first approach, see Stavenga et al., 2006).

Here, we compare measured wing reflectance spectra of various pierid species with the absorption spectra of pterins extracted from the wings.

2. Materials and methods

2.1. Animals

Various members of the Pieridae were photographed and spectrophotometrically investigated in the collections of the Entomology Department, Agricultural University (Wageningen, Netherlands), in the National Museum of Natural History, Naturalis (Leiden, the Netherlands), and in the Royal Museum of Central Africa (Brussels, Belgium). The specimens used in the extraction experiments were captured at various locations (*Delias nigrina* in Australia, *Gonepteryx rhamni*, *Pieris brassicae* and *Anthocharis cardamines* in the Netherlands, by D.G.S., and *Colias erate* and *P. r. crucivora* in Japan, by Dr. K. Arikawa); other specimens (see Table 1) were obtained from commercial suppliers.

2.2. Photography

A Nikon D70 digital camera, which has a red channel with substantial UV sensitivity, was used to photograph the specimens. For UV photography, a 15 W blacklight was used, and the camera lens (AF Micro Nikkor, 60 mm, 1:2.8 D) was fitted with UV-transmission and red-blocking filters (3 mm UG1 and BG38; Schott, Darmstadt, Germany). The light source for RGB photography was the Nikon flash unit Speedlight SB-800. The camera axis and illumination was more or less perpendicular to the wing plane.

2.3. Spectrophotometry

Reflectance spectra of intact wings were measured with a reflection probe connected to a fiber optic spectrometer

Table 1
Investigated species and their phylogenetic position

Coliadinae
African clouded yellow, <i>Colias electo</i>
Eastern pale clouded yellow, <i>Colias erate</i>
Orange-barred sulfur, <i>Phoebis philea</i>
Brimstone, <i>Gonepteryx rhamni</i>
<i>Colotis</i> group
Autumn-leaf vagrant, <i>Eronia leda</i>
Scarlet tip, <i>Colotis danae</i>
Purple tip, <i>Colotis regina</i>
Great orange tip, <i>Hebomoia glaucippe</i>
Anthocharidini
Orange tip, <i>Anthocharis cardamines</i>
Pierini
Large white, <i>Pieris brassicae</i>
Small white, <i>Pieris rapae</i>
Black jezebel, <i>Delias nigrina</i>

(SD2000, Avantes, Eerbeek, the Netherlands) using a deuterium/halogen light source, with the plane of the wing approximately perpendicular to the probe. A white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA) served as a reference. Transmittance spectra of small drops (volume approximately 20 μ l) of 1% aqueous ammonia, with 10% isopropylalcohol (see, e.g., Morehouse et al., 2007), in contact with a piece of wing were measured via aluminum-coated and black-painted quartz light guides. The transmittance spectra were subsequently converted into absorbance spectra. The magnitude of the latter spectra was somewhat variable, especially in the iridescent wing areas, presumably due to the variable penetration of the solvent. For each of the species of Figs. 5–7, we have performed several extraction experiments, with the criterion that a minimum of two measurements had to give considerable (and consistent) absorbance changes.

3. Results

3.1. Colors and reflectance spectra of pierid wings

The spectral characteristics of pierid butterflies depend on the subfamily, as is already suggested by the common names for the main subfamilies, the sulfurs (Coliadinae) and the whites (Pierinae). Fig. 1 presents the current pierid phylogeny (after Braby et al., 2006). A few examples are given in Fig. 2, which presents ultraviolet (UV, left column) as well as common (RGB, middle column) photographs, together with reflectance spectra (right column) for four male pierids. The top and lower row photographs of each species represent the dorsal and ventral wings, respectively. The dorsal wings of the male African clouded yellow, *Colias electo* (Fig. 2a), are mainly orange, but the orange scales also strongly reflect the ultraviolet, due to

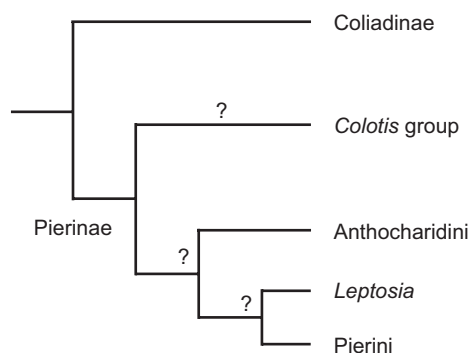


Fig. 1. Phylogeny of the Pieridae (after Braby et al., 2006). Butterflies of the subfamily Coliadinae, or sulfurs, have wings with a characteristic yellow or orange color. The dorsal wings of the males often have areas dominated by short-wavelength iridescence. Members of the *Colotis* group have mainly white or yellow wings, and the males have generally a dorsal wing tip that is orange or red colored and exhibits short-wavelength iridescence. The Anthocharidini have usually white or sometimes yellow wings, and the males have yellow or orange dorsal wing tips that are occasionally iridescent. The Pierini have overall white dorsal wings.

multilayers in the scale ridges (Ghiradella et al., 1972). In the male autumn-leaf vagrant, *Eronia leda* (Fig. 2b), only the wing tips are orange, and the orange scales also exhibit UV iridescence. The main part of the dorsal wings is yellow, which corresponds with a high reflectance at the longer wavelengths and a low reflectance in the shorter wavelength range. This demonstrates the presence of a pigment that absorbs UV as well as violet light. The scarlet tip, *Colotis danae* (Fig. 2c), has red dorsal wing tips with UV iridescence, and the main part of the dorsal wings is white. The white wing reflectance is high in the visible range, but it is low in the ultraviolet, revealing a purely UV-absorbing pigment. The dorsal wings of the male small white, *Pieris rapae rapae* (Fig. 2d), are very white, but also have a very low UV reflectance, indicating a UV-absorbing pigment. UV iridescence is fully absent here.

Compared to the dorsal wings, the ventral wings of all four cases have less bright colors. Ventrally there is no iridescence, and generally the color pattern is more or less cryptic. Resting pierids have usually their wings closed, which suggests that the dorsal wings of males are used for display and signaling, and that the ventral wings rather function to suppress visibility. In most cases female pierids lack iridescence, and have generally less chromatic colorations, which reinforces the idea that male coloration has been more strongly selected in the context of visual signaling (Kemp et al., 2005; Rutowski et al., 2007).

3.2. Extraction of pterin pigments

The shape and amplitude of the reflectance varies with the location on the wing (Fig. 2), which may result from the variation in stacking of the scales and the identity and quantity of pigments within the scales. To further investigate the importance of pterin pigments to wing coloration, we have extracted the pterin pigments by applying small drops of aqueous ammonia. Fig. 3 shows the case of the dorsal wing of a male orange tip, *A. cardamines*. The scales at the dorsal wing tip are non-iridescent in the UV (Fig. 3a and c), contrary to those of the orange tips of the male *E. leda* (Fig. 2b). The two drops of aqueous ammonia applied to the orange tip and the white wing area (Fig. 3a and b) bleach the wing locally (Fig. 3c and d). The drops extract two types of pigment, one absorbing in the UV- and blue-wavelength range, and one absorbing exclusively in the UV.

To study the extraction process more quantitatively, we have put an aqueous ammonia drop in between two coated quartz light guides (Fig. 4). Light from a deuterium/halogen light source was focused into one light guide, and the transmitted light was guided via the second light guide to a photodiode array spectrophotometer (PDA-SP; Fig. 4). The drop made contact with a piece of wing at time $t = 0$, and the transmittance spectrum was then measured every 5 s. Fig. 5a shows the change in transmittance during the extraction process of the yellow dorsal wing of a male brimstone, *G. rhamni*, and Fig. 5b presents the absorbance

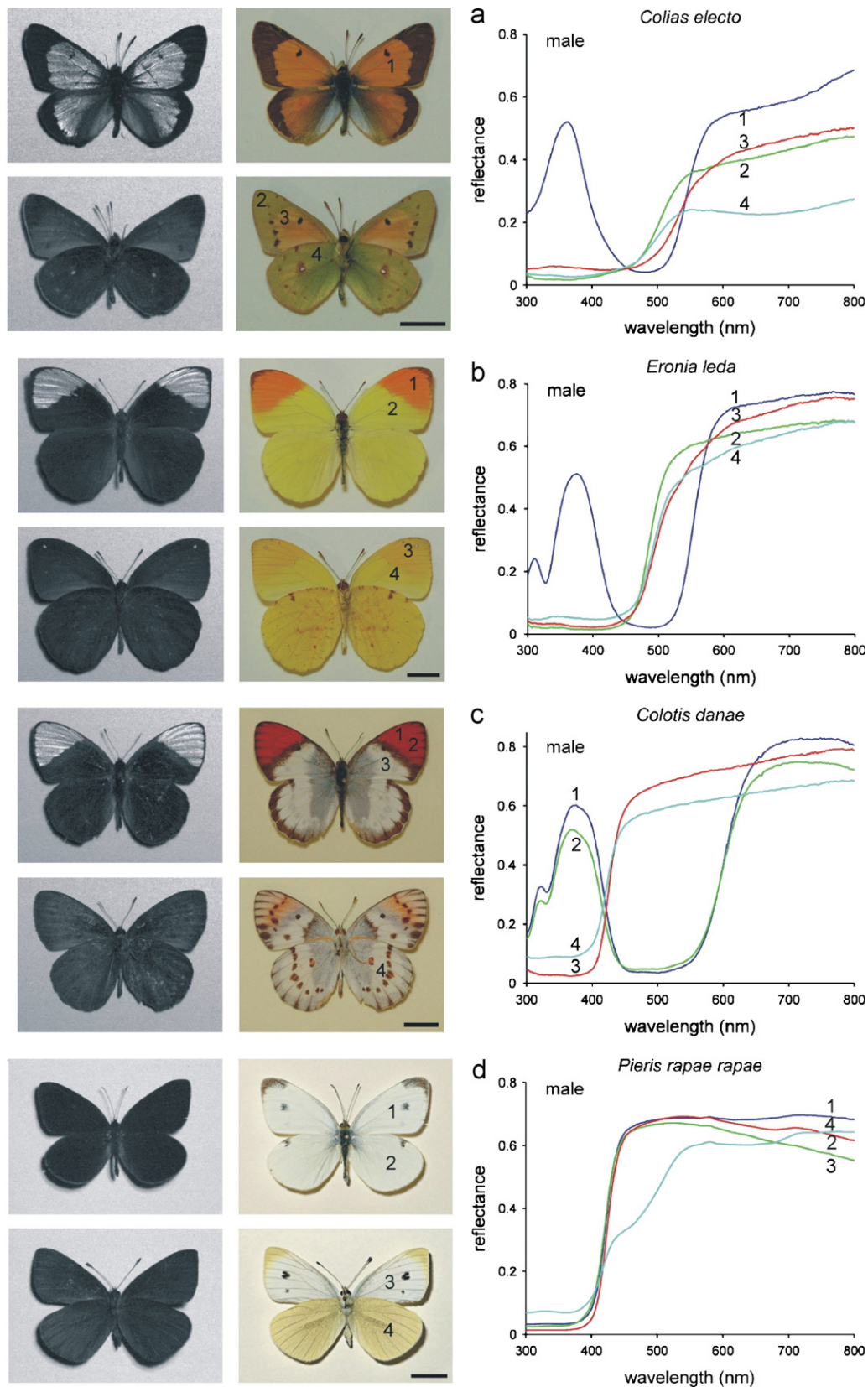


Fig. 2. Photographs and reflectance spectra of four male pierids: African clouded yellow, *Colias electo* (a); autumn-leaf vagrant, *Eronia leda* (b); scarlet tip, *Colotis danae* (c); and small white, *Pieris rapae rapae* (d). The left column presents UV photographs, and the middle column normal RGB photographs. The top row photographs of each species represent the dorsal wings, and the lower row photographs are taken from the ventral wings. The numbers in the photographs indicate the area where the reflectance spectrum (right column) with the corresponding number was measured.

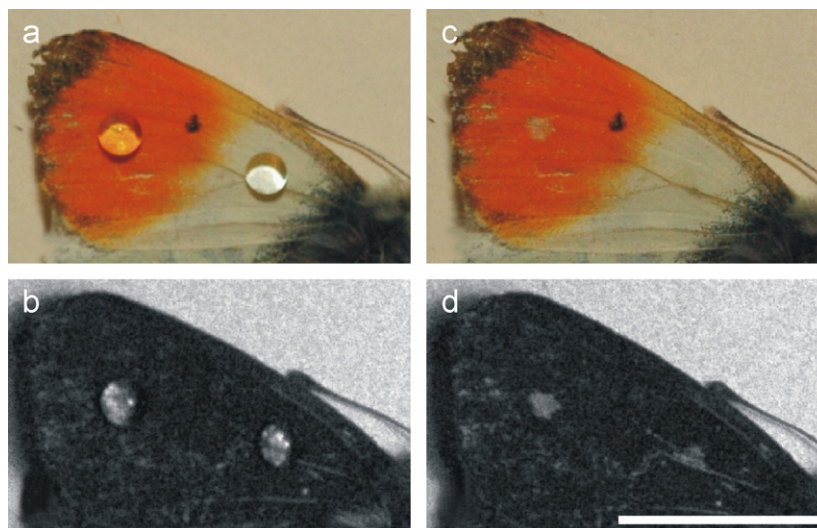


Fig. 3. The left dorsal forewing of the male orange tip, *Anthocharis cardamines*. (a) An RGB photograph, and (b) a UV photograph, where a drop of aqueous ammonia is positioned on the orange wing tip, and another drop is placed in the adjacent white area of the forewing. A few minutes after application, the drops were removed and the photographs of (c) and (d) were taken. The ammonia has extracted two types of pterin pigments located in the orange and UV-absorbing wing areas, respectively. Bar: 1 cm.

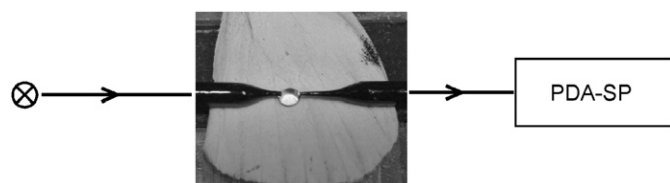


Fig. 4. Diagram of the setup for measuring the absorbance spectra of pterins locally extracted from a pierid butterfly wing (here *Pieris brassicae*). Light from a deuterium/halogen light source is focused into a coated and black-painted quartz light guide, which has a slender tip. The tip of a similar light guide is positioned opposite and close to the first light guide, so that in between the tips a drop of aqueous ammonia can be administered. Part of the light from the first light guide is transmitted by the drop and the second light guide and is channeled into a photodiode array spectrophotometer (PDA-SP). The wing is put into contact with the drop by a micromanipulator.

spectra calculated from the transmittance spectra. The absorbance spectra have two main bands, one in the ultraviolet, with peak wavelength at about 290 nm, and one in the violet, peaking at 390 nm, the amplitude of which increases over time (Fig. 5c). The time courses of the 290 and 390 nm peaks distinctly differ, demonstrating that at least two different pigments are involved. The pigment with the 290 nm peak is extracted more rapidly than the pigment absorbing maximally at 390 nm. The time course of the normalized absorbance in the trough, at 340 nm, is identical to the time course of the normalized absorbance at 390 nm.

Fig. 6 presents similar measurements in the orange dorsal wing tip of a male great orange tip, *Hebomoia glaucippe*. The absorbance spectra show again two main bands, one peaking in the ultraviolet at about 290 nm, and one in the blue wavelength range, peaking at 450 nm. The ultraviolet absorbance peak rises more rapidly than the

blue peak, again demonstrating the extraction of two different pigments. The normalized absorbance at the intermediate shoulder (340 nm) and that at the blue peak (450 nm) rises with the same time course, showing that the shoulder belongs to the blue-absorbing pigment.

We have performed the same procedure on the wings of members of several pierid species. Fig. 7 presents averaged absorbance spectra, normalized to the ultraviolet peak, measured from aqueous ammonia drops applied to different wing locations of a few Coliadinae species (Fig. 7a–c), members of the *Colotis* group and Anthocharidini (Fig. 7d–f), and Pierini (Fig. 7g–i). A dominant ultraviolet peak occurs in all cases. In one case, the female small white, *P. r. crucivora*, the absorbance spectrum features only the ultraviolet band, with a slight side band around 340 nm. Additional bands, depending on the species and wing location, occur at longer wavelengths. In some cases only one additional band exists, for instance the yellow dorsal forewing of the brimstone (Fig. 7a), and in other cases there are two additional bands, for instance the white/yellow ventral wing of the large white (Fig. 7g) and the red and purple tip areas of the two *Colotis* species (Fig. 7d and e).

We have grouped the cases with one band additional to the ultraviolet band around 290 nm, and we have normalized the absorbance spectra to the peak value of the additional band. The absorbance spectra thus assemble in clear sets, with peak wavelengths at approximately 290, 340, 390 and 450 nm, corresponding to white (W), very white (Wh), yellow (Ye) and orange (Or) wing colors, respectively (Fig. 8). The spectrum measured from the dorsal wings of the female *P. r. crucivora* (*P.r.c.f.*) peaks at virtually the same wavelength as the absorption spectrum measured from a drop of uric acid solution

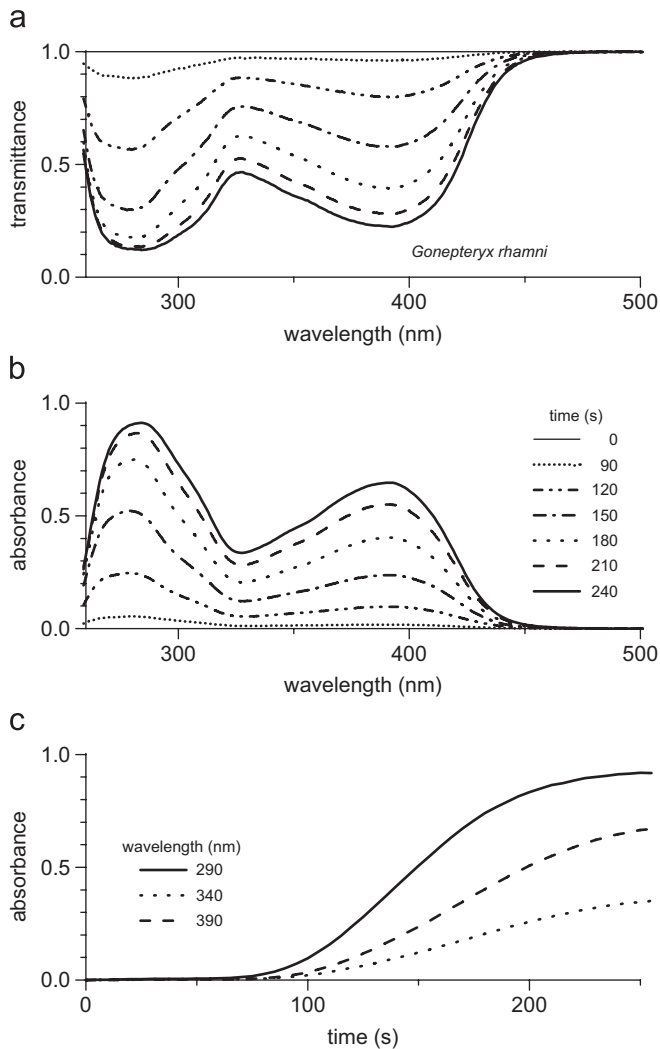


Fig. 5. (a) The change in transmittance during the extraction process of the yellow dorsal wing of a male brimstone, *Gonepteryx rhamni*, measured with the setup of Fig. 4. (b) Absorbance spectra calculated from the series of transmittance spectra of (a). (c) Time course of the absorbance changes at wavelengths 290, 340 and 390 nm, showing that at least two different pigments are extracted. The pigment absorbing maximally around 290 nm is extracted faster than the pigment with absorption peak at 390 nm.

(U.a.), but the absorption band is somewhat wider. The sideband of the spectrum of the female *P. r. crucivora* suggests that the wings contain a slight amount of pigment absorbing at about 340 nm, a pigment that is dominantly present in the white wings of the male *P. r. crucivora* (Fig. 8, *P.r.c.m.*) and *P. brassicae* (Fig. 8, *P.b.*). In the following, we assume that this is leucopterin (although there might be a trace of isoxanthopterin; Makino et al., 1952; Harmsen, 1966; Watt and Bowden, 1966). The same pigment exists in the main, bright-white colored part of the dorsal wings of the male orange tip, *A. cardamines* (Fig. 8, *A.c.*). The pigment with absorption peak at about 390 nm, extracted from the yellow wing areas of the male brimstone, *G. rhamni*, and the orange-barred sulfur, *Phoebis philea* (Fig. 8, *G.r.* and *P.p.*), is probably

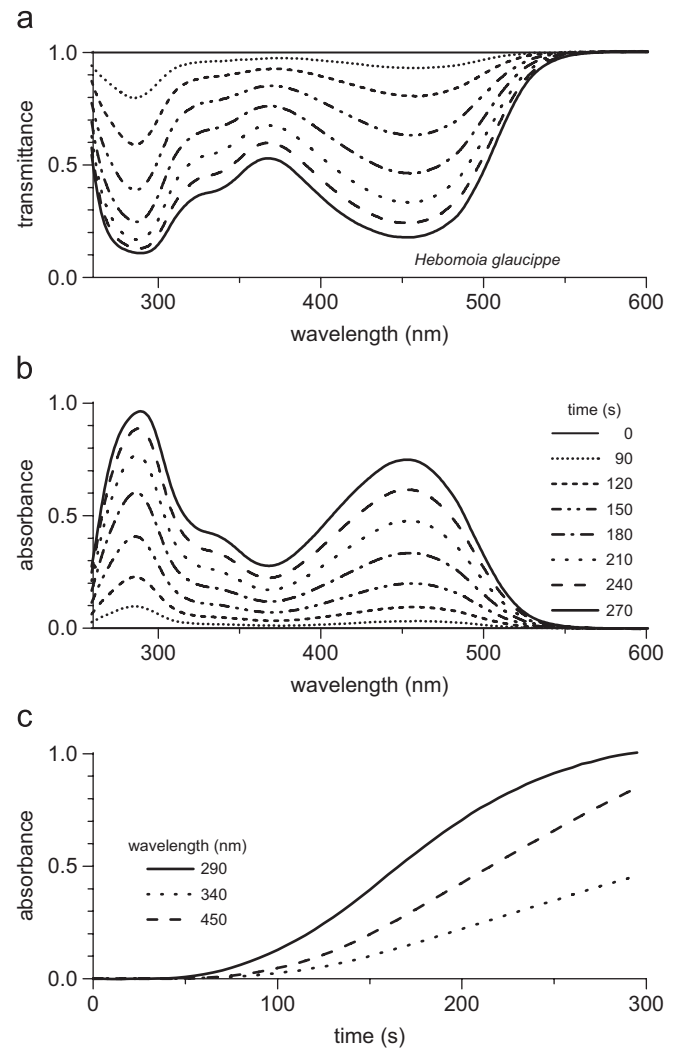


Fig. 6. (a) The change in transmittance during the extraction process of the orange tip of the dorsal forewing of the great orange tip, *Hebomoia glaucippe*. (b) Absorbance spectra calculated from the series of transmittance spectra of (a). (c) Time course of the absorbance changes at wavelengths 290, 340 and 450 nm, demonstrating the extraction of at least two different pigments. The pigment absorbing maximally at 290 nm is extracted considerably faster than the pigment with absorption peak at 450 nm.

xanthopterin (with possibly some dihydroxanthopterine; Watt, 1964; Descimon, 1971). The pigment with absorption peak at about 450 nm, extracted from the orange tips of the male orange tip, *A. cardamines* (Fig. 8, *A.c.*), and the great orange tip, *H. glaucippe* (Fig. 8, *H.g.*), has been characterized as erythropterin (Schöpf and Becker, 1936; Pfeleiderer, 1961).

The absorbance spectra in Fig. 7 consisting of multiple bands, for instance those from the red tip of the male *C. danae* (Fig. 7d) and the purple tip of the male *Colotis regina* (Fig. 7e), can be explained as to be due to mixtures of the pigments with absorption spectra peaking at 340 and 450 nm (leucopterin and erythropterin). Apparently, the 450 nm pigment (erythropterin) can cause both orange and red wing colors (Pfeleiderer, 1961, 1962).

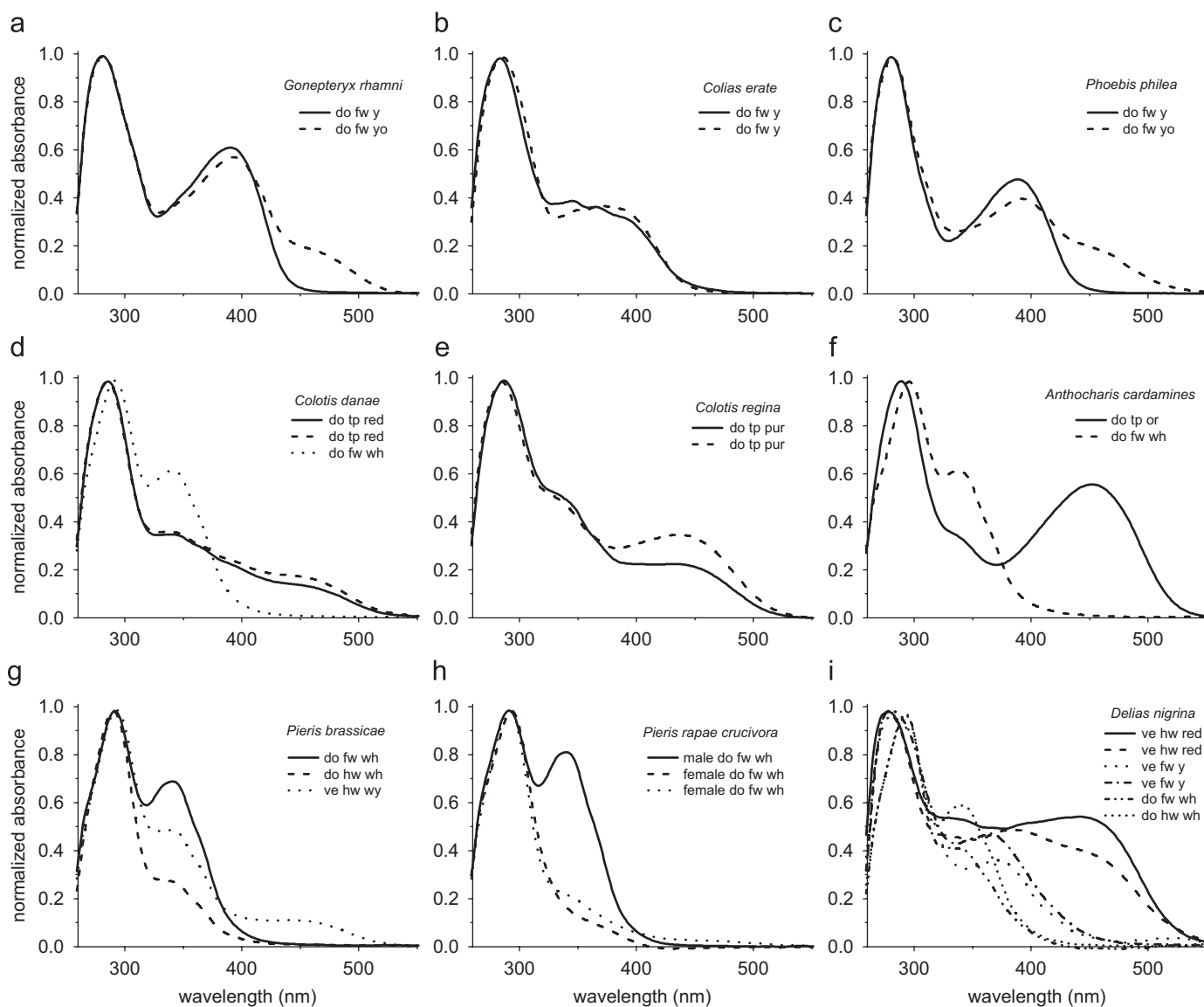


Fig. 7. Absorbance spectra, normalized to the ultraviolet peak, measured from aqueous ammonia drops applied to different wing locations of three Coliadinae species (a–c), members of the *Colotis* group and Anthocharidini (d–f), and Pierini (g–i). The butterflies were all males, except for the female (feml) *Pieris rapae crucivora*. The spectra were measured from the dorsal (do) or ventral (ve) forewing (fw) or hindwing (hw), or from the dorsal tip (tp), of wing areas colored yellow (y), yellow/orange (yo), red (red), purple (pur), orange (or), white (wh) or white/yellow (wy).

3.3. Pigment absorbance and wing reflectance spectra

We thus come to the question of whether the reflectance spectra of Fig. 2 can be understood with the set of pigment absorbance spectra of Fig. 8. For instance, the reflectance spectra measured from the white wing areas of *P. r. rapae* (Fig. 2d, #1–3) and *C. danae* (Fig. 2c, #3, 4) have approximately a constant amplitude for wavelengths above 450 nm, but the reflectance is minor at wavelengths below 400 nm. It is commonly accepted that the latter is due to the pterin pigment that can be extracted from white wings and that absorbs in the near UV, which we presume to be leucopterin. The measured reflectance spectrum should hence be immediately understandable from the measured pigment absorption spectrum.

To put this question more explicitly, we have assembled in Fig. 9a the reflectance spectra of white wing areas of male *H. glaucippe* (*H.g.*), *P. r. crucivora* (*P.r.c.m.*), *C. danae* (*C.d.*), *A. cardamines* (*A.c.*) and *P. brassicae* (*P.b.*). The amplitudes at the longer wavelengths vary somewhat, which can be easily understood, because microscopical observations readily show that the density of the scale stacks can vary considerably among species and even wing area (see Stavenga et al., 2006). The wavelength range where the reflectance rapidly rises, around 425 nm, is virtually constant, however, clearly suggesting the action of one and the same pigment.

Fig. 9b gives two reflectance spectra for yellow wing areas of the male brimstone (*G.r.*), together with reflectance spectra from yellow wing areas of *P. philea* (*P.p.*) and

E. leda (*E.l.*; see Fig. 2b, #2). One curve of the brimstone features a reflectance band in the UV, which is due to the interference reflectors of the scale ridges. The other reflectance spectra of Fig. 9b are low in the ultraviolet and blue wavelength range up to 450 nm, presumably due to absorbing pigment, xanthopterin. The rise in reflectance of all four spectra of Fig. 9b occurs around 480 nm.

Fig. 9c is an accumulation of reflectance spectra measured from orange and red wing areas. The spectra of the male *C. electo* (*C.e.*; see also Fig. 2a), *H. glaucippe* (*H.g.*), *C. danae* (*C.d.*; Fig. 2c, #1) and *E. leda* (*E.l.*; Fig. 2b, #1) have a band in the ultraviolet, peaking around 360 nm, due to iridescent scales; the reflectance spectrum of *C. regina* (*C.r.*) has an iridescence band in the blue, peaking around 500 nm. The short-wavelength iridescence

is superimposed on a low reflectance due to pigment absorption. In the orange scales, the reflectance rises steeply at about 560 nm, but the half-maximal reflectance of the red and purple tips of *C. danae* and *C. regina* is at about 600 and 630 nm, respectively. The reflectance spectrum of the red bands of the ventral wings of the black jezebel, *D. nigrina* (*D.n.*), is located in between the reflectance spectra of the two *Colotis* species. The spread of the reflectance spectra of orange and red wing areas, from which one and the same pigment (erythropterin; Pfeleiderer, 1963; Descimon, 1971) was extracted, is discussed below.

4. Discussion

Two main optical mechanisms determine the coloration of pierid wings, light scattering and light absorption. Incoherent light scattering always occurs, that is, a main component of the reflection is randomly scattered light, because the scale structures are arranged more or less non-periodically. Pigments, acting as long-pass filters, reduce the scattered light flux depending on the pigments' absorption spectrum, thus creating a colored appearance of the wings. In our extraction experiments, we have classified three different pigments with main absorption bands in the UV, violet and blue wavelength range, and we identified them with the three main pterins of pierids: leucopterin, xanthopterin and erythropterin (Fig. 8). The extracts may have contained minor additions of iso-xanthopterin and dihydroxanthopterin, or other members of the pterin families (Harmsen, 1966; Descimon, 1975). A dominant component, extracted most rapidly from the investigated pierids' wings, absorbs maximally around 290 nm, similar as uric acid (Fig. 8). Uric acid has been reported for numerous pierids (Harmsen, 1966; Tojo and Yushima, 1972), but it is irrelevant for our analysis of the wing colors, however, as it absorbs light of a wavelength

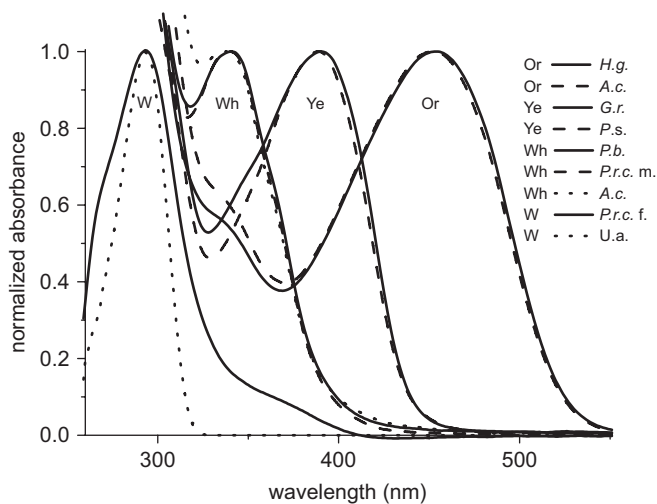


Fig. 8. Absorbance spectra of Figs. 5–7 with two main bands normalized to the peak of the longest wavelength band, together with the single band spectra measured from the dorsal wing of a female *Pieris rapae* r. *crucivora* (*P.r.c.f.*) and a solution of uric acid (*U.a.*).

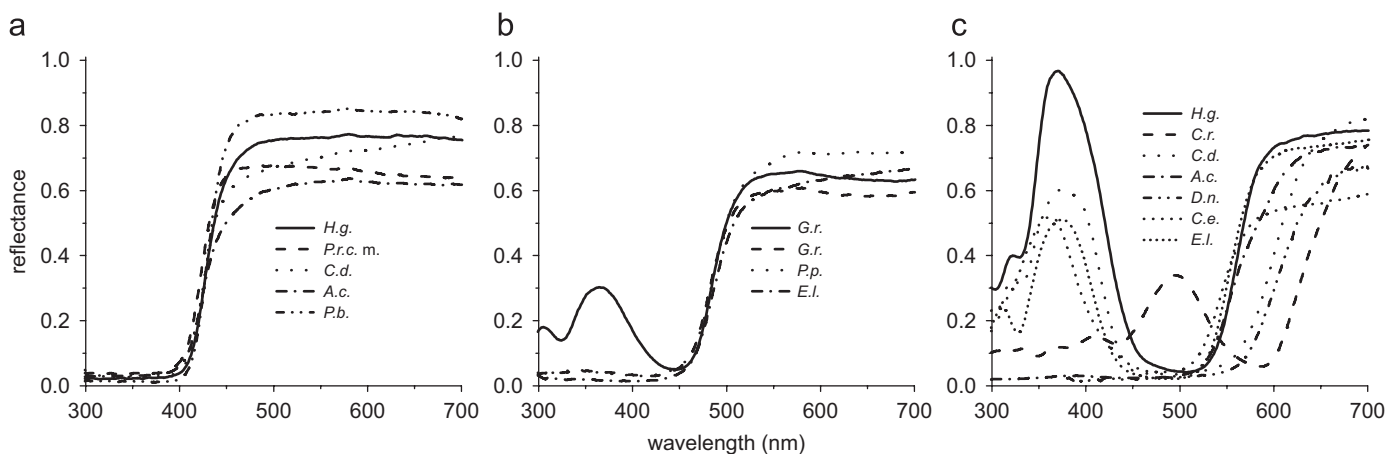


Fig. 9. Reflectance spectra measured from various pierid butterflies. (a) White wing areas of a male *Hebomoia glaucippe* (*H.g.*), *Pieris rapae crucivora* (*P.r.c.m.*), *Colotis danae* (*C.d.*), *Anthocharis cardamines* (*A.c.*) and *Pieris brassicae* (*P.b.*). (b) Yellow wing areas of *Gonepteryx rhamni* (*G.r.*), *Phoebis philea* (*P.p.*) and *Eronia leda* (*E.l.*). (c) Orange and red wing areas of *H. glaucippe* (*H.g.*), *Colotis regina* (*C.r.*), *C. danae* (*C.d.*), *A. cardamines* (*A.c.*), *Delias nigrina* (*D.n.*), *Colias electo* (*C.e.*) and *E. leda* (*E.l.*). The white and yellow areas have virtually identical reflectance spectra, except for the differences in amplitude and the additional UV peak of *G. rhamni*. The reflectance spectra of orange wing areas are similar, but the spectra of the red areas obviously deviate from those of the orange areas. See the text for further discussion.

range that is not present in nature, and is outside the visible wavelength range, including that of butterflies.

Coherent scattering, causing iridescence, is encountered in the dorsal wings of males of many pierid species (Silberglied and Taylor, 1973; Kemp et al., 2005; Rutowski et al., 2007). A survey of reflectance spectra shows that the iridescence emerges as a distinct band, with bandwidth <100 nm, peaking virtually always in the UV-violet wavelength range. Only in a few cases, the iridescence band peaks at blue wavelengths, e.g., the male purple tip, *C. regina* (Fig. 9c). The iridescence band is easily recognized in the reflectance spectrum, as it is superimposed on a low level of reflectance resulting from the pterin pigment, which acts as a long-pass filter on the incoherently scattered light; the remaining long-wavelength reflectance band is always well separated from the iridescence band. The iridescence peak wavelength is presumably flexible, as it is determined, together with the bandwidth, by the spacing of the multilayers in the scale ridges (Land, 1972). The absorption bands of the different types of pterins must be rigid, however. Indeed, the coincident reflectance spectra of the white and yellow wings (Fig. 9a and b) agree with the extraction of two distinct pigments from white and yellow wings, leucopterin and xanthopterin.

The pterins cause the low reflectance of the white and yellow wing areas in the shorter wavelength range. However, the detailed optical mechanism underlying the

low reflectance appears to be non-trivial. It is clear that incident light is reflected as well as transmitted by the wing scales, and because the scales are stacked in overlapping layers on both sides of the wings, multiple reflections and transmissions in the various scale layers determine the total wing reflectance. A detailed optical analysis of the small white, *Pieris rapae*, demonstrated that wing reflectance and transmittance are both very minor in the UV, due to pigment absorption that is extreme in the UV and falls off in the wavelength range 400–450 nm (Stavenga et al., 2006). The latter study suggests that we should be able to estimate the spectral shape of the wing pigments by observing the measured reflectance spectra (see also Watt, 1968). We therefore processed the reflectance spectra of Fig. 9 as follows. After removing the iridescence band, if present, the remaining reflectance spectrum was normalized to its maximal value, and then subtracted from 1. Fig. 10 presents the resulting absorption spectra together with the corresponding pterin spectra, which were taken from Fig. 8. It thus appears that the absorption spectra deduced from the reflectance spectra have a slope similar to that of the pterin absorption spectra, but the spectra are separated by a broad wavelength gap.

The reflectance of the pigmented wings is very low in the short-wavelength range, which suggests that the spectral gap might be the direct consequence of the high optical density of the pterins. To test that possibility, Fig. 11 shows the spectral absorption, $A(\lambda)$, by a homogeneous medium

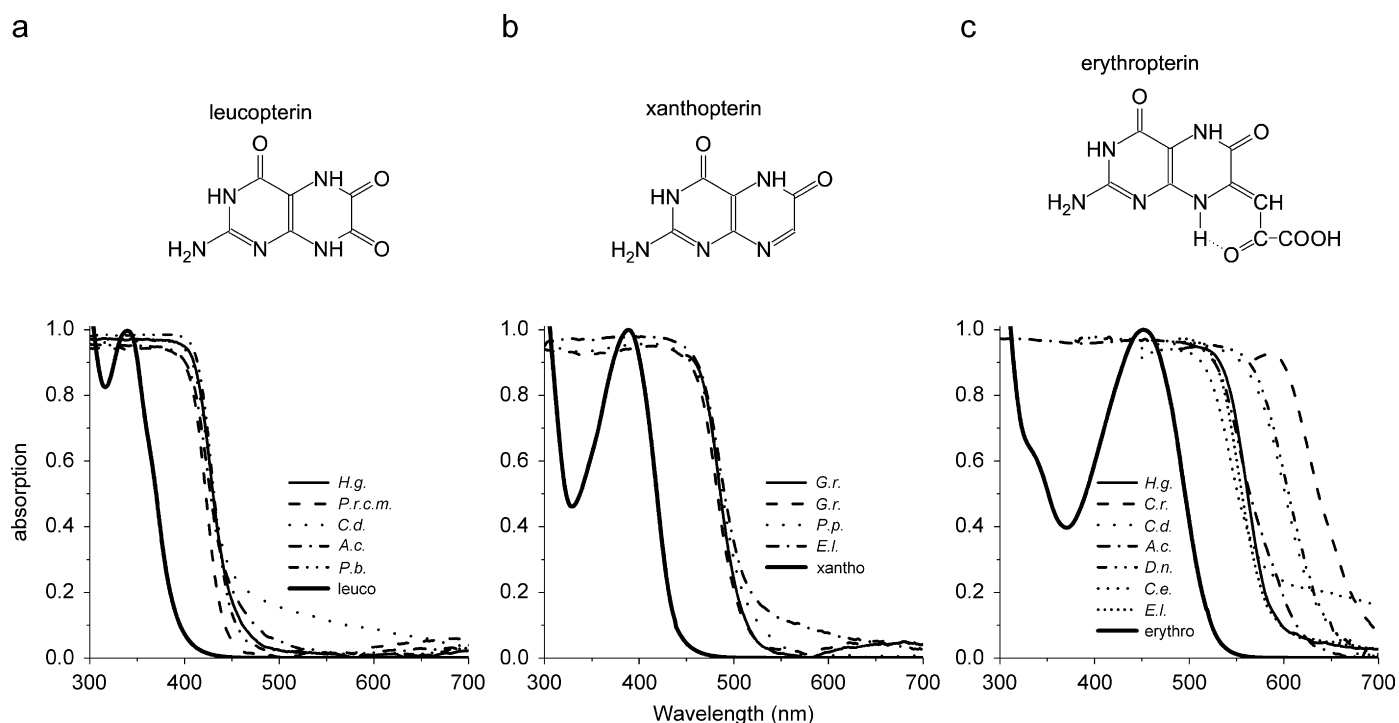


Fig. 10. Normalized absorption spectra of leucopterin (a, leuco), xanthopterin (b, xantho) and erythropterin (c, erythro) compared with absorption spectra derived from the reflectance spectra of Fig. 9a–c (for species abbreviations, see legend of Fig. 9). The latter absorption spectra were derived from the spectra in Fig. 9 by first neglecting the iridescence band, and then subtracting the normalized reflectance spectrum from 1. The pterin spectra were taken from Fig. 8: for the leucopterin spectrum that of the male *Pieris rapae crucivora* (*P.r.c.m.*); for the xanthopterin spectrum that of the male brimstone, *Gonepteryx rhamni* (*G.r.*); and for the erythropterin spectrum that of the male great orange tip, *Hebomoia glaucippe* (*H.g.*).

containing xanthopterin, with maximal optical densities of $n = 0.5, 1, 2, 4, 8$ and 16 (at 390 nm), calculated using Lambert–Beer’s law, with $A(\lambda) = 10^{-n\kappa(\lambda)}$, where λ is the wavelength, and $\kappa(\lambda)$ is the normalized absorption coefficient given by the xanthopterin spectrum. For comparison, Fig. 11 also shows one of the absorption spectra following from the reflectance measurements (*G.r.*, the normalized, dashed brimstone spectrum of Fig. 10b). It so appears that a density of >30 is necessary for the absorbing medium to approach the absorption spectrum inferred from the brimstone reflectance spectrum.

Of course, the scale medium is far from homogeneous, and thus light traveling through the scales will have covered a variety of path lengths when it is eventually leaving the wing scales as scattered light. The effective absorption spectrum will therefore be a weighted average of absorptions associated with the different paths. Taking this all into account, it is very difficult to conceive that the majority of scattered light will have traveled paths with optical densities larger than about 30 (that is 30 log units of absorbance). The spectral gap between the xanthopterin spectrum and the absorption spectra inferred from the yellow wing reflectance spectrum thus seems unbridgeable. The same holds for the gap between the leucopterin spectrum and the absorption spectra deduced for the white wings of Fig. 10a. An even more problematic situation exists for the erythropterin spectrum and the absorption spectra concluded for the red wing areas, where the spectral gap is much wider (Fig. 10c). The latter case thus strongly suggests that the pterin in red wings differs spectrally from the extracted erythropterin. Actually, the same conclusion appears to be inescapable for

all cases. We have to note that the absorption spectra of pterins depend on the pH, and that the pH in the scale will not be identical to that of aqueous ammonia, but the published pH-dependent shifts are no more than 20 nm, and therefore the spectral gaps must be due to other causes (Pfleiderer, 1962; Melber and Schmidt, 1992). The most likely hypothesis to overcome the deviation between extracted and in situ spectra is that the pterins are spectrally modified when bound to and/or compacted within the scale granules. This could result from the formation of a dimer, like pterorhodin, which has been stated to be responsible for red-colored wings (Descimon, 1971), or the cause could be a possible binding protein, but so far we do not have evidence for one or another possibility. We conclude that a quantitative explanation of the wing colors of pierid butterflies requires further study, concerning both the physical optics as well as the pigment chemistry.

The coloration of the various pierids appears to be related to the phylogeny (Fig. 1), which has been recently studied into great detail (Braby et al., 2006). Pigmentary colors are seen in all pierid wings, even in the white wings of males of the *Pieris* species. The white color seen by humans, which is purely due to incoherent scattering, suggests the absence of pigment. (The black spots or black stripes are due to scales pigmented by melanin, but this pigment is not concentrated in granules; Stavenga et al., 2004.) Male white *Pieris* are nevertheless highly colored for the butterflies, as they acutely see UV light. Female *P. r. crucivora* will be ‘butterfly white’ as the reflectance of their wings is approximately constant at all wavelengths (Obara and Majerus, 2000; Giraldo and Stavenga, 2007). Male *P. r. crucivora* hence easily discriminate males from females (Obara, 1970). The Japanese *P. r. crucivora* is an outstanding example within the pierid tribe Pierini, as the European *P. r. rapae* only has a slight sexual dichromatism (Obara and Majerus, 2000; Giraldo and Stavenga, 2007), and in other species the difference between the sexes is mainly recognized from the number of melanic spots.

In another tribe, the Anthocharidini (Fig. 1), sexual dichromatism is well-known, as the males have strongly colored orange dorsal wing tips, as in *A. cardamines* (Fig. 3; but sometimes the tip is yellow, as in *A. scolymus*), in addition to the mainly white colored wings. Some species are polymorphic and can have mainly yellow wings (*A. cethura*; Scott, 1986). The sexual dichromatism of the Anthocharidini is generally achieved through sex-dependent differences in pigmentary coloration, but the males of some species also feature iridescence in the wing tips (*A. sara*; Scott, 1986).

Coherent scattering causing iridescent dorsal wing tips in males is a virtually universal property of members of the *Colotis* group. The color of the males’ dorsal wing tips is orange or red, highly contrasting with the color of the main part of the dorsal wings, which usually is white (Fig. 2c), but also can be yellow (Fig. 2b). The females of the *Colotis* group generally exhibit no iridescence and are much less prominently colored than the males.

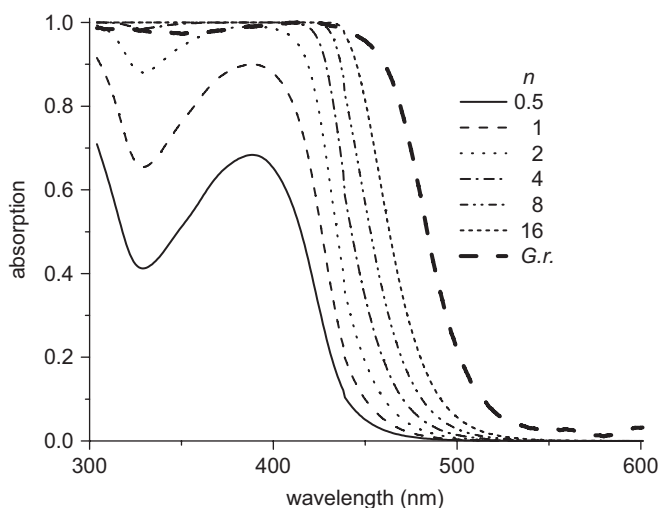


Fig. 11. Absorption spectra of a xanthopterin solution with optical densities $n = 0.5, 1, 2, 4, 8$ and 16 , calculated using Lambert–Beer’s law, with $A(\lambda) = 10^{-n\kappa(\lambda)}$, where λ is the wavelength and $\kappa(\lambda)$ is the normalized absorption coefficient given by the xanthopterin spectrum (xantho; see Fig. 10b). The bold dashed curve is the normalized absorption spectrum derived from the wing reflectance of a male brimstone, *Gonepteryx rhamni* (*G.r.*; see dashed curve in Fig. 10b). The latter absorption spectrum can only be approximated with Lambert–Beer’s law for unrealistic high xanthopterin pigment densities.

Ultraviolet iridescent wings with yellow or orange pigmentary colors are the hallmark of male Coliadinae, although there are clear exceptions (Kemp et al., 2005). In some species, as *C. electo*, the wings of both male and female are similar orange with black margins (Fig. 2a). Only the male is UV iridescent. In other species, the wing color can also be sex-dependent, as in *G. rhamni*, where the male's overall iridescent dorsal wings are yellow, whilst the female wings are white, but with low reflectance in the UV. In its relative, *G. cleopatra*, the male dorsal wings are mainly yellow, but a large part of the dorsal forewing is orange, and that area is also UV iridescent.

Figs. 1 and 2 suggest an evolutionary trend in coloration of the Pieridae (Stavenga and Arikawa, 2006). Only certain Coliadinae feature a prominent, overall orange color, which is due to a pigment that absorbs over a wide wavelength range, presumably erythropterin. This pigment is also responsible for the coloration of the orange or red dorsal wing tips of members of the *Colotis* group and the Anthocharidini. Xanthopterin, presumably responsible for the yellow color displayed by the whole wing of many Coliadinae species, also exists in members of the *Colotis* group and in the Pierini, although not prominently. Leucopterin, which absorbs exclusively in the UV, determines the appearance of the Pierini and the white wing parts of *Colotis* species, and even the white wings of females of certain Coliadinae. When going from the Coliadinae to the Pierini, parallel to the retreat of the pigment's absorption spectrum towards shorter wavelengths, the abundance of short-wavelength iridescence progressively decreases. The latter phenomenon is quite understandable, because by adding UV iridescence the color signal of an orange wing is enhanced (Rutowski et al., 2005), but adding UV iridescence to a white wing with low UV reflectance would achieve the opposite; it would reduce the color signal.

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